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For: SLIDE STAINER WITH HEATING

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DECLARATION OF RON ZEHEB, PH.D., UNDER 37 C.F.R. § 1.132

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Sir:

I, Ron Zeheb, Ph.D. of 134 Rosemont Drive, N. Andover, MA 01845, hereby declare that:

1. I am the Director of Diagnostic Molecular Pathology at The Lahey Clinic in Burlington, MA. In that position, I oversee histological procedures at The Clinic.

2. I have extensive experience in using, developing, and improving stainers and staining technology. I have over 10 years of experience in developing and improving stainer and staining technology.

3. For a total of four years, I worked for Ventana Medical Systems, Inc. in Tucson, Arizona, first as a Senior Research and Development Scientist for 2 years and then as the Director of Research and Development for 2 years. As part of my duties at Ventana Medical Systems, Inc., I oversaw the development of microscope slide stainers and chemistries for automation of *in situ* hybridization and immunohistochemical assays.

4. For a total of five years, I worked for CytoLogix Corporation in Cambridge, Massachusetts first as the Director of Research and Development for 2 years and then as the Vice President of Research and Development for 3 years. As part of my duties at CytoLogix Corporation, I developed and launched an automated slide staining system and managed the development of histological stains for use on an automated slide-staining platform. As a former employee of CytoLogix Corporation, I own a very small amount of stock in the company.

5. For two years, I worked for DakoCytomation Corporation in Cambridge, Massachusetts, first as Vice President, then Senior Vice President of Research and Development. I was also the General Manager with responsibilities for all operations in Cambridge, Massachusetts. I managed all the research and development activities in the United States, including the development of stainers for use in histochemistry protocols.

6. In May 1994, the time of the filing of U.S. Application No. 10/823,368, one of ordinary skill would not have predicted the utility of plural heated surface areas, each heated by an electric heater thereunder and having a respective temperature sensor, in a random access dispensing assembly or in a method for processing biological samples mounted on microscope slides.

7. In May 1994, the time of the filing of U.S. Application No. 10/823,368, to my knowledge all staining processes could have been categorized as either routine staining or advanced staining.

8. To my knowledge, routine staining was performed as a batch process where all slides were treated the same. The slides were typically mounted in baskets that were dipped into buckets of solution. As such, they did not require random access dispensing systems as claimed. Further, they generally did not require heating.

9. To my knowledge, there are three general categories of advanced staining, commonly known as special stains, immunohistochemistry, and *in situ* hybridization.

10. In May 1994, the time of filing of U.S. Application No. 10/823,368, special stain techniques often required judgments on the part of the technician, such as color analysis. Namely, the technician dipped the slide in a chemical or dye until the tissue elements acquired a certain specified color, as determined visually. Examples of special stain processes are presented Luna (1968) (Exhibit A), Prophet et al. (1992) (Exhibit B) and Bancroft and Stevens (1996) (Exhibit C). In these references, arrows with asterisks indicate steps in the procedures which must be performed visually and thus require user input. Because such techniques rely highly on the skills of the technician, and are considered an art, they had not been considered appropriate for automatic processing.

11. The second type of advanced stain is immunohistochemistry. As practiced in 1994, immunohistochemical slides were either processed at room temperature (without the application of heat) or were heated to approximately body temperature. In either situation, all of the slides were processed at the same temperature, regardless of the particular immunohistochemical stain. The automated slide stainers on the market by 1994 did one or the other. Examples of automated slide stainers without any heating capability were Fisher's Code-on and Shandon's Cadenza; whereas, Ventana's 320/ES immunohistochemical slide stainer heated all of the slides to approximately body temperature.

12. The third category of advanced staining is *in situ* hybridization (ISH). This type of stain requires temperatures that are much higher than body temperature, often in the 70-95°C range. However, the small volume of reagent probe typically used (approximately ten microliters) can

rapidly evaporate at such temperatures. To prevent evaporation during ISH processing, the tissue section and the small amount of reagent/probe are sealed on the slide using a coverslip. The edges of the coverslip are sealed with, for example, rubber cement or nail polish. A system in which the sample must be sealed off from the outside environment is mechanically incompatible with a device that controls relative movement between a liquid dispenser and a platform, and that dispenses liquid reagents onto a microscope slide bearing a biological sample, such as in the claimed dispensing assembly. One of ordinary skill at the time of the invention (1994) would not have considered a system with high temperature heating and a stringent requirement for preventing evaporation of an extremely low volume of reagent as compatible with an open dispensing system.

13. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Ron Zeheb, Ph.D.

10/12/07

Date

# Manual of HISTOLOGIC STAINING METHODS of the Armed Forces Institute of Pathology

**Third Edition**

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EXHIBIT
A
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MANUAL OF HISTOLOGIC STAINING METHODS
OF THE ARMED FORCES INSTITUTE OF PATHOLOGY

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18. Rinse thoroughly in three changes of absolute alcohol. It is essential not to use low grade alcohols. If low grade alcohols are used, the cytoplasmic stains are dissolved and the tissue will not take on the collagen stain, which is made up in absolute alcohol.

19. Alcoholic safran solution for 5-15 minutes.

20. Dehydrate in three changes of absolute alcohol, and clear in several changes of xylene. If the collagen is not sufficiently yellow, repeat the staining with safran.

21. Mount with Permount or Histoclad.

RESULTS

| | |
|---|---|
| Nuclei | -black |
| Elastic fibers | -dark purple to black |
| Collagen and reticulum fibers | -yellow |
| Ground substance | -blue to bluish green |
| Fibrinoid | -intense red |
| Muscle | -red |

REFERENCE. Movat, H. Z.: *Arch. Path.*, 60:289-295, 1955.

## JONES' METHOD FOR KIDNEY

FIXATION. 10% buffered neutral formalin, Bouin's or Zenker's

TECHNIQUE. Cut paraffin sections at 2 microns.

SOLUTIONS

### 0.5% PERIODIC ACID SOLUTION

(See page 72)

### 3% METHENAMINE* SOLUTION

| | |
|---|---|
| Hexamethylenetetramine (methenamine) | 3.0 gm |
| Distilled water | 100.0 ml |

### 5% SILVER NITRATE SOLUTION

(See page 91)

### BORATE BUFFER SOLUTIONS (STOCK)

#### Solution A: 0.2 *M* Boric Acid

| | |
|---|---|
| Boric acid | 12.36 gm |
| Distilled water | 1000.0 ml |

#### Solution B: 0.05 *M* Sodium Borate

| | |
|---|---|
| Sodium borate | 19.07 gm |
| Distilled water | 1000.0 ml |

*Fisher Scientific Co. or Eastman Kodak Co.

### BORATE BUFFER SOLUTION, pH 8.2 (WORKING)

Solution A ..... 6.5 ml
Solution B ..... 3.5 ml

### 1% GOLD CHLORIDE SOLUTION (STOCK)

(See page 90)

### GOLD CHLORIDE SOLUTION (WORKING)

Gold chloride stock solution ..... 10.0 ml
Distilled water ..... 40.0 ml

Solution is stable for approximately 100 slides.

### 3% SODIUM THIOSULFATE (HYPO) SOLUTION

Sodium thiosulfate ..... 3.0 gm
Distilled water ..... 100.0 ml

### METHENAMINE SILVER SOLUTION, pH 8.2 (WORKING)

Methenamine, 3% ..... 42.5 ml
Silver nitrate, 5% ..... 2.5 ml
Borate buffer, pH 8.2 ..... 12.0 ml

Prepare fresh just before use and filter. This solution is stable for approximately 60-75 minutes. After this time, there is a breaking down process, which produces a black precipitate and is picked up on the slides.

STAINING PROCEDURE. Chemically clean glassware must be used.

*Note.* It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chloride free distilled water. Distilled water may be checked for free chloride by the addition of several drops of 5% silver nitrate solution. If a white cloud appears upon the addition of the silver nitrate, discard the sample of water and replace.

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 11 minutes.
3. Rinse in chloride free distilled water.
4. Filter freshly prepared methenamine-silver solution into coplin jar.
5. Place slides in methenamine-silver solution and then place coplin jar in pre-warmed 70 °C water bath. Start timing at this point, approximately 60-75 minutes. Check under microscope when slides show macroscopically a medium brown color.

*Note.* Solution and slides should be allowed to come to 70 °C together. While slides are in the silver solution they may be examined after they begin to show macroscopically a medium brown color reaction. Before checking under the microscope, they are first rinsed in hot 70 °C chloride free distilled water, checked, and then returned to hot water rinse and then returned into hot staining solution. Slides should be checked every 10 minutes when they have reached the dark or medium brown stage. Slides should be checked as rapidly as possible because if the section cools there is an un-

even staining of the section. When the desired staining time has been reached, the slide should be checked as described above, every 1-2 minutes. Strict adherence to the timing is essential in order to obtain a uniform consistency in staining. A properly stained section at this point should have a dark brownish-yellow background; the reticulum fibers will be intense black, as should the basement membranes. An overstained section will be too black. Differentiation will be very difficult as the black will be so intense as to obscure many or all of the tissue elements. The section may be destained with an extremely dilute solution of potassium ferricyanide for one or two dips.

6. Rinse section well in distilled water.
7. Tone in working gold chloride solution for 1 minute.

*Note:* If sections are overtoned, place in 3% sodium metabisulfite for 1-3 minutes, checking periodically.

8. Rinse well in distilled water.
9. Sodium thiosulfate solution for 1-2 minutes.
10. Wash in running tap water for 10 minutes.
11. Rinse well in distilled water.
12. Counterstain with routine Harris hematoxylin and eosin stain.
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, three changes each.
14. Mount with Permount or Histoclad.

## RESULTS

| | |
|---|---|
| Basement membrane | - black |
| Reticulum fibers | - black |
| Nuclei | - blue |
| Cytoplasm, collagen, and connective tissue | - pink to orange |

REFERENCE. Jones, D. B.: *Amer. J. Path.* 27:991-1009, 1951. Modified by Avalone, F., G. U. Branch, Armed Forces Institute of Pathology.

Armed Forces Institute of Pathology

# Laboratory Methods in Histotechnology

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# VERHOEFF'S ELASTIC STAIN

FIXATION: 10% buffered neutral formalin or any other well-fixed tissue.

SECTIONS: Paraffin, 6 micrometers.

## SOLUTIONS

10% ALCOHOLIC HEMATOXYLIN SOLUTION (Ch. 3)

10% FERRIC CHLORIDE SOLUTION (Ch. 3)

### VERHOEFF'S IODINE SOLUTION

| | |
|---|---|
| Iodine | 2.0 gm |
| Potassium iodide | 4.0 gm |
| Distilled water | 100.0 ml |

Mix the crystals of iodine and the crystals of iodide in a flask. Shake vigorously. Then gradually add the distilled water, 20 ml at a time.

### VERHOEFF'S ELASTIC STAIN WORKING SOLUTION

| | |
|---|---|
| Alcoholic hematoxylin, 10% | 25.0 ml |
| Alcohol, 100% ethyl | 25.0 ml |
| Ferric chloride, 10% | 25.0 ml |

Mix well, then add:

| | |
|---|---|
| Verhoeff's iodine solution | 25.0 ml |

### 2% FERRIC CHLORIDE DIFFERENTIATING SOLUTION

| | |
|---|---|
| Ferric chloride, 10% | 20.0 ml |
| Distilled water | 80.0 ml |

VAN GIESON SOLUTION (Ch. 3)

5% SODIUM THIOSULFATE (HYPO) SOLUTION (Ch. 3)

## PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Stain in Verhoeff's elastic stain working solution for 15 minutes.
3. Wash in lukewarm running tap water for 20 minutes.
4. Place in distilled water.
5. Differentiate in 2% ferric chloride solution. Check microscopically.[a] Elastic fibers are black and sharply fined; the background is gray.
6. Place in 5% sodium thiosulfate solution for 1 minute.
7. Wash in tap water for 5 minutes.
8. Place in distilled water.
9. Counterstain in van Gieson solution for 1 minute.[b]

10. Dehydrate rapidly[c] through 95% ethyl alcohol (2 changes) and absolute ethyl alcohol (2 changes); clear in 2 changes of xylene.
11. Mount with resinous medium.

## RESULTS

| | |
|---|---|
| Elastic fibers | black |
| Nuclei | black |
| Collagen | red |
| Other tissue structures | yellow |

[a]Wipe the back of the slide. While wet with the 2% ferric chloride differentiation solution, check under low power. Elastic fibers in arterial walls should be black and the arterial wall muscle, gray.

[b]Do not leave in van Gieson solution for more than 1 minute. The picric acid component decolorizes the elastic fibers.

[c]Rinse rapidly in 95% ethyl alcohol to avoid decolorizing the van Gieson solution.

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## REFERENCE

Mallory FB. *Pathological Technique.* Philadelphia, PA: WB Saunders; 1942:170-171.

## LEVADITI-MANOVELIAN METHOD FOR SPIROCHETES

FIXATION. 10% buffered neutral formalin. Specimen should be 1 mm thick.

TECHNIQUE. Embed in paraffin after staining is completed (see Staining Procedure Step 10).

### SOLUTIONS

#### 3% SILVER NITRATE SOLUTION

| | |
|---|---|
| Silver nitrate | 3.0 gm |
| Distilled water | 100.0 ml |

#### REDUCING SOLUTION

| | |
|---|---|
| Pyrogallic acid | 4.0 gm |
| Formalin, 37 – 40% | 5.0 ml |
| Distilled water | 100.0 ml |

### STAINING PROCEDURE

1. Rinse specimen in tap water, after fixation.
2. Let stand in 95% alcohol for 24 hours.
3. Transfer to distilled water and leave until the tissue sinks to the bottom of the container.
4. Place in freshly prepared silver nitrate solution and keep in 37°C in the dark for 3 to 5 days, changing the solution three times.
5. Rinse in distilled water.
6. Reducing solution at room temperature, in the dark for 24 to 72 hours.
7. Rinse in distilled water.
8. Dehydrate in 80% alcohol, 95% alcohol, and absolute alcohol, two changes, 30 minutes each.
9. Clear in oil of cedarwood for two changes, 1 hour each and infiltrate with two changes of paraffin 45 minutes each.
10. Embed in paraffin.
11. Cut sections at 5 microns and mount on slides.
12. When dry, deparaffinize with xylene, three changes.
13. Mount with Permount or Histoclad.

### RESULTS

| | |
|---|---|
| Spirochetes | - black |
| Background | - yellow to light brown |

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 293.

## WARTHIN-STARRY METHOD FOR SPIROCHETES AND DONOVAN BODIES

FIXATION. 10% buffered neutral formalin. *Avoid chromate fixatives.*

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS. Use chemically clean glassware.

### ACIDULATED WATER

Triple distilled water .....1000.0 ml
Add enough 1% aqueous citric acid to bring water to pH 4.0.

### 1% SILVER NITRATE SOLUTION (For impregnation)

Silver nitrate, C.P. crystals .....1.0 gm
Acidulated water .....100.0 ml

### 2% SILVER NITRATE SOLUTION (For developer)

Silver nitrate, C.P. crystals .....2.0 gm
Acidulated water .....100.0 ml

### 5% GELATIN SOLUTION

Sheet gelatin, high grade .....10.0 gm
Acidulated water .....200.0 ml

### 0.15% HYDROQUINONE SOLUTION

Hydroquinone, crystals, photographic quality .....0.15 gm
Acidulated water .....100.0 ml

Keep 2% silver nitrate, 5% gelatin, and 0.15% hydroquinone in 50 ml Erlenmeyer flasks, in a flotation bath at 54 °C until developer is made.

### DEVELOPER SOLUTION

Silver nitrate solution, 2% .....1.5 ml
Gelatin solution, 5% .....3.75 ml
Hydroquinone solution, 0.15% .....2.0 ml

Combine in the order given in small beaker, making certain solutions are mixed well. *Prepare immediately before use.*

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to triple distilled water.
2. Impregnate with silver nitrate solution heated in a flotation bath to 43 °C for 30 minutes. Prepare the developer solution at this point. (See *Note*)
3. Flood sections, that have been laid across glass rods, with the developer solution which *must be used as soon as it is mixed.* Allow sections to develop until they are light brown or yellow. Check known control under the microscope. The spirochetes should be black and the background light brown or yellow. (See *Note*)
4. Wash quickly and thoroughly in hot tap water, approximately 56 °C.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

| | |
|---|---|
| Spirochetes, Donovan bodies | - black |
| Background | - pale yellow to light brown |

REMARKS. It may be necessary to prolong development of sections for the demonstration of Donovan bodies. Certain hematogenous pigments, nuclei and melanin have a greater attraction for silver than do spirochetes, and it is difficult to stain the spirochetes in close proximity to these elements. By lowering the pH of the acidulated solution to 3.6 and prolonging the development, the spirochetes may be demonstrated in the areas of competition; however, the part of the section not containing competitive elements may be overstained and useless. Sections can be restained to increase the amount of development if microscopic observation of the known positive tissue shows pale spirochetes or none at all.

*Note*. Use paraffin coated forceps, particularly at step 2 and step 3.

REFERENCES. Kerr, D. A.: *Amer. J. Clin. Path. Tech. Suppl.* 8:63-67, 1938. Copyright by Williams and Wilkins Co. (AFIP modification)

C. H. Bridges, and L. G. Luna studied permissible variations of this technic in the AFIP laboratories. Their report can be found in *Lab. Invest.* July-August, 1957.

5. Differentiate in 95% alcohol until blue ceases to come out into alcohol and erythrocytes and collagen are pink.

6. Dehydrate in absolute alcohol, clear in xylene, two changes each.

7. Mount with Permount or Histoclad.

RESULTS

| | |
|---|---|
| Nuclei | - blue |
| Basophile leucocyte, mast cell granules | - purple to violet |
| Cartilage | - purple |
| Erythrocytes, eosinophile granules | - pink |
| Cytoplasm | - blue to pink |

REFERENCE. Mallory, F. B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 196.

## MAY-GRUNWALD GIEMSA METHOD

FIXATION. Zenker's or other well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

### JENNER SOLUTION (STOCK)

| | |
|---|---|
| Jenner stain, dry powder* | 1.0 gm |
| Alcohol, methyl | 400.0 ml |

### JENNER SOLUTION (WORKING)

| | |
|---|---|
| Jenner solution (stock) | 25.0 ml |
| Distilled water | 25.0 ml |

### GIEMSA SOLUTION (STOCK)

(See page 119)

### GIEMSA SOLUTION (WORKING)

| | |
|---|---|
| Giemsa solution (stock) | 50 drops |
| Distilled water | 50.0 ml |

Make fresh, do not re-use.

### 1% GLACIAL ACETIC WATER SOLUTION

(See page 94)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).

*National Aniline Certified

3. Wash in running water for 10 minutes.
4. Rinse in distilled water, two changes.
5. Methyl alcohol, two changes for 3 minutes each.
6. Working Jenner solution for 6 minutes.
7. Working Giemsa solution for 45 minutes.
8. Handle each slide individually in this and subsequent steps. Differentiate in glacial acetic-water solution then check microscopically for well differentiated nuclei.
9. Rinse in distilled water.
10. Dehydrate quickly in 95% alcohol, absolute alcohol, and clear with xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

| | |
|---|---|
| Nuclei | - blue |
| Cytoplasm | - pink to rose |
| Bacteria | - blue |

REFERENCE. Strumia, M. M.: *J. Lab. Clin. Med.* 21:930-934, 1935-1936.

## MALLORY'S METHOD FOR HEMOFUCHSIN

FIXATION. Zenker's solution, absolute alcohol or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

### ALUM HEMATOXYLIN SOLUTION

| | |
|---|---|
| Hematoxylin | 1.0 gm |
| Aluminum ammonium or potassium sulfate | 20.0 gm |
| Distilled water | 400.0 ml |
| Thymol | 1.0 gm |

### 0.5% BASIC FUCHSIN SOULTION

| | |
|---|---|
| Basic fuchsin | 0.5 gm |
| Alcohol, 95% | 50.0 ml |
| Distilled water | 50.0 ml |

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Alum hematoxylin solution until the nuclei stand out sharply.
3. Wash thoroughly in water.
4. Basic fuchsin solution for 30 minutes.
5. Wash in water.
6. Differentiate in 95% alcohol until hemofuchsin granules stand out sharply against a gray background.
7. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

# Theory and Practice of Histological techniques


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*Notes*

a. The blue counterstain may be patchy if extensive caseation is present. Care should be taken not to over-counterstain as scant organisms can easily be obscured in this way.
b. Decalcification using strong acid can destroy acid fastness; formic acid is recommended.
c. Victoria blue can be substituted for carbol fuchsin and picric acid for the counterstain if colour blindness causes a recognition problem.

## Cold ZN method for tubercle bacilli (Kinyouin, 1915)

*Sections*

Formalin-fixed, paraffin.

*Staining solution*

| | |
|---|---|
| Basic fuchsin | 4 g |
| Phenol crystals | 5 g |
| 95 per cent alcohol | 20 ml |
| Distilled water | 100 ml |

Dissolve the basic fuchsin in the alcohol, and mix with the phenol and distilled water. Filter and add 1 drop of Teepol to every 30 ml of the solution.

*Method*

1. Take sections to water.
2. Stain in filtered carbol fuchsin solution at room temperature, 20 min.
3. Wash in tap water, and differentiate in 1 per cent acid alcohol, controlling microscopically.
4. Wash in tap water, 5–10 min.
5. Counterstain in 0.2 per cent methylene blue, 30 seconds.
6. Blot, dehydrate, clear and mount in DPX.

*Results*

As for standard technique

## Fluorescent method for tubercle bacilli (Kuper & May, 1960)

*Sections*

Formalin-fixed, paraffin.

*Staining solution*

| | |
|---|---|
| Auramine O | 1.5 g |
| Rhodamine B | 0.75 g |
| Glycerol | 75 ml |
| Phenol crystals (liquified at 50°C) | 10 ml |
| Distilled water | 50 ml |

*Method*

1. Take section to water (using a mixture of 1 part groundnut oil and 2 parts xylene to remove wax for *M. leprae*).
2. Pour on preheated staining solution, filtered and at 60°C, 10 min.
3. Wash in tap water.
4. Differentiate in 0.5 per cent hydrochloric acid in alcohol for *M. tuberculosis*, or 0.5 per cent aqueous hydrochloric acid for *M. leprae*.
5. Wash in tap water, 2 min.
6. Quench background fluorescence in 0.5 per cent potassium permanganate, 2 min.
7. Wash in tap water and blot dry.
8. Dehydrate (not for *M. leprae*), clear and mount in a fluorescence-free mountant.

*Results*

Using blue light fluorescence (below 530 nm)
Tubercle or leprosy bacilli — *golden yellow*
Background — *dark green*

*Notes*

a. The advantage of increased sensitivity of this technique is offset by the inconvenience of setting up the fluorescence microscope.
b. Preparations fade over time, depending on their exposure to UV light.

## Wade–Fite technique for leprosy bacilli (Wade 1957, modified)

*Sections*

Paraffin, formalin-fixed

*Solutions*

As for ZN technique

*Method*

1. Warm the sections and dewax using a mixture of 1 part groundnut oil or clove oil and 2 parts xylene to remove wax, 10 min.
2. Repeat blotting and washing in water until section is uniformly wetted

## Gram method for bacteria in smears

*Method*

1. Fix the dry film by passing it three times through a flame.
2. Stain with 1 per cent crystal violet or methyl violet, 15 seconds, then pour off the excess stain.
3. Flood with Lugol's iodine, 30 seconds, then pour off excess.
4. Flood with acetone for not more than 2–5 seconds; wash with water immediately. Alternatively, decolorise with alcohol until no more stain comes away; wash with water.
5. Counterstain with dilute carbol fuchsin, 20 seconds, or neutral red (freshly filtered) 1–2 min.
6. Wash with water and blot dry.

*Results*

Gram-positive organisms — *blue-black*
Gram-negative organisms — *red*

## Gram stain for paraffin sections (Gram, 1884)

*Sections*

Formalin-fixed, paraffin.

*Solutions*

*a. Crystal violet solution:*
0.5 per cent crystal violet in 25 per cent alcohol.

*b. Gram's and Lugol's iodine*

| | |
|---|---|
| Iodine | 1 g |
| Potassium iodide | 2 g |
| Distilled water | 10 ml |

Shake or grind until dissolved, make up to 300 ml, with distilled water, for Gram's iodine or 100 ml for Lugol's iodine.

*c. 1 per cent aqueous neutral red*

*Method*

1. Take sections to water
2. Stain with filtered crystal violet solution, 2 min.
3. Rinse in tap water and drain.
4. Pour on the iodine solution b, 2 min.
5. Rinse in tap water, blot and flood with acetone, 1–2 seconds.
6. Wash in tap water.
7. Counterstain in neutral red, 3 min.
8. Blot, dehydrate rapidly, clear and mount in DPX.

*Results*

Gram-positive organisms, fibrin, some fungi, Paneth cell granules, keratohyalin and keratin — *blue*
Gram-negative organisms — *red*

## Gram-Twort stain (Twort, 1924; Ollet, 1947)

*Solutions*

*a. Crystal violet solution*
As for previous method.

*b. Gram's iodine*
As for previous method.

*c. Twort's stain*

| | |
|---|---|
| 0.2 per cent neutral red in ethanol | 9 ml |
| 0.2 per cent fast green in ethanol | 1 ml |
| Distilled water | 30 ml |

Mix immediately before use.

*Method*

1. Dewax in xylene, hydrate through graded alcohols to water.
2. Stain in crystal violet solution, 3 min.
3. Rinse in running tap water.
4. Treat with Gram's iodine, 3 min.
5. Rinse in tap water, blot dry, and complete drying in a warm place.
6. Differentiate in preheated acetic alcohol (2 per cent acetic acid in absolute alcohol) at 56°C until no more colour washes out. This may take 15–20 min; the section should be a light brown or straw colour.
7. Rinse briefly in distilled water.
8. Stain in Twort's stain, 5 min.
9. Wash in distilled water.
10. Rinse in acetic alcohol, until no more red leaves the section; this should take only a few seconds.

11. Rinse in clean alcohol, clear in xylene and mount in DPX.

*Results*
Gram-positive organisms — *blue-black*
Gram-negative organisms — *pink to red*
Nuclei — *red*
Red blood cells and most cytoplasmic structures — *green*
Elastic fibres — *black*

*Note*
Twort's stain can be used with effect as a counterstain in the basic method on p. 294, instead of neutral red. Again the green counterstain facilitates the detection of the red-staining Gram-negative organisms.

## TECHNIQUES FOR MYCOBACTERIA

These organisms are difficult to demonstrate by the Gram technique because they possess a capsule containing a long-chain fatty acid, mycolic acid, which makes them hydrophobic. This fatty capsule influences the penetration and resistance to removal of stain by acid and alcohol (acid- and alcohol-fast), and is of variable robustness between the various species which make up this group. Phenolic acid and, frequently, heat are used to reduce the surface tension, increasing porosity and forcing dyes to penetrate this capsule. The speed of removal by differentiation with acid/alcohol of the primary dye is proportional to the extent of the fatty coat. The avoidance of defatting agents such as alcohol and xylene in methods for *M. leprae* are an attempt to conserve its fragile fatty capsule.

Mycobacteria are PAS-positive due to the carbohydrate present in their cell walls. This positivity is only evident when large concentrations of the organisms are present. When organisms die they lose their fatty capsule and consequently their ZN positivity. The carbohydrate can still be demonstrated by the Grocott methenamine silver reaction which may prove useful when the ZN fails, particularly if the patient is already receiving TB therapy.

A possible source of contamination may be found growing in the glutinous material lining some taps and connected rubber tubing. These organisms are acid- and alcohol-fast but are usually easily identified as contaminants by their appearance as clumps above the focal plane of the section, i.e. floaters.

### Ziehl–Neelsen stain for tubercle bacilli (1882, 1883)

*Fixation*
Formalin or any except Carnoy's

*Section*
Paraffin

*Reagents required*

*a. Carbol-fuchsin*
1 g of basic fuchsin is dissolved in 10 ml of absolute alcohol, and 100 ml of 5 per cent aqueous phenol is added. Mix well. Filter before use.

*b. Acidified methylene blue*
0.25 per cent methylene blue in 1 per cent acetic alcohol.

*Method*
1. Dewax in xylene and hydrate through graded alcohols to water.
2. Flood section with freshly filtered carbol fuchsin and heat to steaming (by intermittent flaming), 15 min, OR stain in a Coplin jar at 56–60°C (oven or water bath), 30 min.
3. Wash well in tap water.
4. Differentiate in 1 per cent acid alcohol, 10 min.
5. Wash in tap water, 5–10 min.
6. Counterstain in methylene blue solution, 30 seconds.
7. Blot, and differentiate by alternate dehydration and rehydration until the background is a delicate pale blue.
8. Finally dehydrate, clear and mount in DPX.

*Results*
Tubercle bacilli, hair shafts, Russell bodies, Splendore–Hoeppli immunoglobulin around Actinomyces and some fungal organisms — *red*
Background — *pale blue*